Journal of Physiology (1990), **426**, *pp.* 499–520
With 12 figures
Printed in Great Britain

MODULATION OF INWARDLY RECTIFYING CHANNELS BY SUBSTANCE P IN CHOLINERGIC NEURONES FROM RAT BRAIN IN CULTURE

BY K. YAMAGUCHI*, Y. NAKAJIMA†, S. NAKAJIMA‡ AND P. R. STANFIELD§

From the Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

(*Received 27 November 1989*)

SUMMARY

1. Whole-cell recording was used to investigate the effects of substance P on cultured neurones from the rat nucleus basalis.

2. Brief applications of substance P produced a reduction, about 1 min in duration, of resting membrane conductance. The concentration producing a half-maximal effect was approximately 40 nM, with the continuous presence of substance P resulting in desensitization of the response.

3. The control current–voltage relation exhibited inward rectification over the voltage range −70 to −150 mV, and hyperpolarization produced a time-dependent decrease of current (inactivation).

4. The substance P-sensitive current, obtained by subtracting the current during the presence of the tachykinin from the control current, showed no time-dependent inactivation, though its current–voltage relation also revealed inward rectification, with the reversal potential being approximately equal to the potassium equilibrium potential, V_K.

5. The relation between the substance P-sensitive chord conductance and voltage could be fitted by a Boltzmann equation, with changes in $[K^+]_o$ shifting this relation along the voltage axis roughly in parallel with the shift in V_K. The maximum conductance was proportional to $([K^+]_o)^{0.7}$.

6. Cs^+ (0·1 mM) blocked the substance P-sensitive current in a voltage-dependent manner, with an equivalent valency for Cs^+ of 1·9. Barium blockage of the substance P-sensitive current was less voltage dependent.

7. Replacement of external Na_+ by tetramethylammonium (TMA^+) ions reduced the substance P-sensitive current by only 18%.

8. These results indicate that substance P inhibits potassium channels with inward rectifier properties very similar to those of skeletal muscle.

* Present address: National Institute for Physiological Sciences, Myodaiji, Okazaki, 444 Japan.
† Present address: Department of Anatomy and Cell Biology, University of Illinois College of Medicine at Chicago, 808 S. Wood Street, Chicago, IL 60612, USA.
‡ Present address: Department of Pharmacology, University of Illinois College of Medicine at Chicago, 835 S. Wolcott, Chicago, IL 60612, USA.
§ Present address: Department of Physiology, University of Leicester, Leicester LE1 7RH.

MS 8102

9. Application of sodium nitroprusside did not alter the effect of substance P, suggesting that cyclic GMP plays no role in the channel modulation.

INTRODUCTION

The tachykinin substance P is widespread in the central and peripheral nervous systems (Nilsson, Hökfelt & Pernow, 1974; Brownstein, Morz, Kizer, Palkovits & Leeman, 1976). Among the neurones that receive synaptic contact from substantial numbers of substance P-containing nerve terminals are the cholinergic neurones of the basal forebrain nuclei (Bolam, Ingham, Izzo, Levey, Rye, Smith & Wainer, 1986; Beach, Tago, & McGreer, 1987), neurones that give a diffuse projection to cerebral cortex. These basal forebrain nuclei, particularly the nucleus basalis of Meynert, are known to degenerate in senile dementia of Alzheimer type (Coyle, Price & DeLong, 1983).

In a previous paper, we have reported that, as in certain other nerve cells (Nicoll, Schenker & Leeman, 1980; Otsuka, Konishi, Yanagisawa, Tsunoo & Akagi, 1982; Adams, Brown & Jones, 1983), substance P produces a long-lasting increase in excitability of the cholinergic neurones from the nucleus basalis by effecting a reduction in the resting potassium permeability (Nakajima, Nakajima, Obata, Carlson & Yamaguchi, 1985). In nucleus basalis neurones, the potassium permeability reduced is that associated with inward rectification (Stanfield, Nakajima & Yamaguchi, 1985). The present paper gives further evidence for the modulation of inward rectification by substance P, describes the biophysical properties of the modulated channels including the blocking effect of certain cations, and presents an account of preliminary experiments concerned with identifying the nature of the second messenger system involved in linking the receptors for substance P with the ion channels modulated.

METHODS

Cell culture

The methods used were essentially the same as those described by Masuko, Nakajima, Nakajima & Yamaguchi (1986) and by Nakajima *et al.* (1985). Newborn Long–Evans rats (1–4 days old) were anaesthetized with ether and their brain stems were removed. Brain slices (400 μm thick) were cut using a vibratome (Lancer 1000), and small tissue fragments containing nucleus basalis were subsequently excised under a dissecting microscope. After treating the brain fragments with trypsin (0·25% trypsin in Ca^{2+}-, Mg^{2+}-free balanced salt solution, 15 min at 37 °C), they were dissociated by trituration in culture medium. The culture medium contained: MEM (80%; GIBCO), L-glutamine (0·29 mg/ml), D-glucose (6 mg/ml), $NaHCO_3$ (3·7 mg/ml), L-ascorbic acid (10 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), fetal bovine serum (10%; GIBCO), and heat-inactivated horse serum (10%; GIBCO). In later experiments we modified the procedure as follows: first neurones were dissociated using papain (12–20 units/ml; Leifer, Lipton, Barnstable & Masland, 1984; Nakajima, Nakajima, Leonard & Yamaguchi, 1986) instead of trypsin, and secondly the culture medium contained 5% heat-inactivated rat serum (prepared in our laboratory) and 10% heat-inactivated horse serum (GIBCO) instead of the combination of fetal bovine and horse serum. These modifications yielded cultured neurones which responded very consistently to substance P.

The dissociated neurones were plated in a small well made at the centre of a Petri dish (O'Lague, Potter & Furshpan, 1978). Before the neurones were plated, the bottom of the well was coated with collagen and with a feeder layer of glial cells. Cultures were kept at 37 °C in an atmosphere of 10% CO_2 in air for 8–20 days. The present experiments were performed on large neurones with a mean

soma diameter of 26·8 ± 0·3 μm (mean ± S.E.M., $n = 85$). Most of these neurones are cholinergic neurones, a previous study (Nakajima *et al.* 1985) having shown that about 75% of large neurones (20 μm or larger in soma diameter) in this culture are cholinergic.

Electrophysiology

The whole-cell version of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The patch pipettes were made from soft glass capillaries (haematocrit tubings; Fisher), which were thoroughly washed, and their tips were fire-polished but not coated with insulating material. The standard pipette solution contained (mM): potassium aspartate, 120; NaCl, 40; $MgCl_2$, 3; EGTA, 0·5; $CaCl_2$, 0·25; Na_2ATP, 2; and HEPES/KOH buffer, 5; KOH, ~ 6; pH 7·2; osmolality 310 mosmol/kg). For a Na^+-free pipette solution, NaCl was replaced by KCl, and Na_2ATP was replaced with K_2ATP. The standard external solution contained (mM): NaCl, 146; KCl, 5; $CaCl_2$, 2·4; $MgCl_2$, 1·3; D-glucose, 11; HEPES/NaOH buffer, 5; tetrodotoxin, 0·001 (pH 7·4; osmolality, 310 mosmol/kg). The procedures for electrophysiological recording from cultured neurones were similar to those described by O'Lague *et al.* (1978). A stainless-steel ring was inserted into the culture dish; the central hole of this steel ring and the centre well of the culture dish constituted the experimental chamber (volume ~ 0·5 ml), with the oxygenated external solution being superfused at a rate of 1–2 ml/min.

The whole-cell currents were measured using a List EPC-7 patch clamp amplifier. The signals were stored on FM tape and later analysed by a digitizer–computer combination. In later experiments, the data were digitized at 5 kHz and analysed automatically by a PDP-11/23 computer. The membrane capacity was neutralized by the cancellation circuit incorporated in the List amplifier, but series resistance was not compensated. The series resistance was estimated from the capacitative transient recorded at high frequency using patch electrodes with Sylgard coating. The ratio of the series resistance and electrode resistance was 1·9 ($n = 5$). From this ratio we estimated the series resistance in the experiments of Figs 4 and 6 to be about 6 MΩ. The average input resistance of our neurones was 104 MΩ in 2·5 mM-K^+ solution. Hence, because of the series resistance the membrane would have been clamped at a voltage 6% smaller than the command pulse (although the value of resting potential is not influenced by series resistance). When the experiments were done in 10 mM-K^+ solution, this error would increase to 10%, and when a large (80 mV) hyperpolarizing pulse was applied in 10 mM-K^+ solution, the membrane would have been clamped at a voltage 17% less than the command voltage pulse. We present our data without correcting for the errors from series resistance and discuss the errors when necessary. The liquid junction potential between the pipette solution and the external solution was 9 mV (external solution positive) at 30 °C, and our data have been corrected for junction potential.

Substance P was applied by pressure ejection through a thoroughly washed capillary (tip diameter, ~ 5 μm) onto the neuronal soma. In earlier experiments the ejection pressure was mostly 0·2–1·3 lbf/in² (1·4–9 kPa) and the distance between the tip of the drug capillary and the edge of the soma was about 10 μm; in later experiments the pressure was mostly 1·0–1·5 lbf/in² (7·0–10 kPa) and the distance 10–20 μm. In the experiments using two different concentrations of substance P, special precaution was necessary: when two drug pipettes were placed near each other, the content of one can contaminate the content of the other pipette. This was probably due to a negative pressure in the drug electrode caused by capillarity. We avoided this contamination by placing both pipettes in the air during intervals between applications, and only when the drug was applied to the cell was one of the pipettes submerged into the bathing solution. Cells with no sensitivity or very weak sensitivity to substance P were discarded. Whenever a good culture run was made, the cells showed consistently good sensitivity. The temperature of the external solution near the neurone, measured by a fine thermocouple (50 μm diameter, Omega Engineering, Inc.), was kept at 29–34 °C (mean, 32 °C).

RESULTS

Responses to a brief application of substance P

In Fig. 1*Aa* a cultured neurone under whole-cell clamp was subjected to depolarizing and hyperpolarizing square-wave pulses once every 2 s, the substance P-containing pipette was brought near the cell, and substance P was ejected by

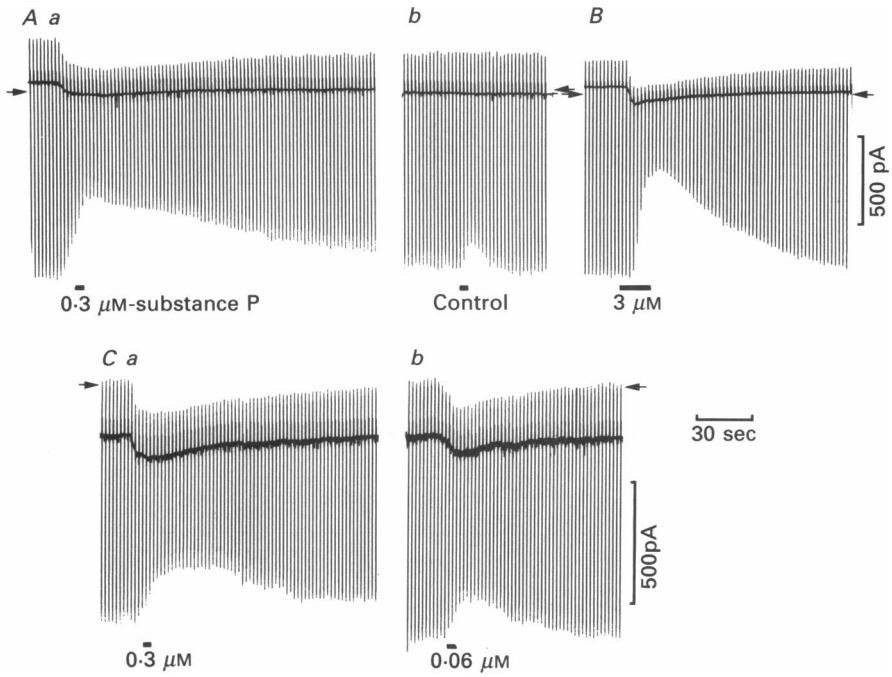

Fig. 1. Substance P application produced a slow inward current and a decrease in membrane conductance. The external solution was 5 mM-K^+ Krebs containing tetrodotoxin (1 μM), and a cell was whole-cell voltage clamped. *Aa*, currents produced by intermittent voltage pulses: each pulse consisted of a square-wave depolarization (18 mV, 100 ms) followed by a 100 ms interval and a square-wave hyperpolarization (57 mV, 100 ms). The holding potential was −74 mV. Arrows indicate the zero current level. The bottom bar indicates the period when substance P (0·3 μM) was applied: the substance P was pressure-ejected using three 1 s duration pulses, each ~ 1 lbf/in² (7 kPa), from a pipette placed ~ 20 μm from the edge of the soma. *Ab*, a control experiment showing an artifact. Same cell as in *Aa*. The procedures were the same as in *Aa*, except that the pipette contained no substance P. The drug pipettes were initially located in the air. Just before the ejection, one pipette was dipped into the solution and brought near the soma. After the end of ejection, it was again raised into the air. Note that in *Aa* the drug effect starts to appear before the start of the pressure-ejection owing to leak of substance P from the pipette. *B*, a different cell. Substance P concentration, 3 μM. The procedures were the same as in *Aa*, except that substance P was applied through a large tip pipette (10 μm diameter) with a smaller pressure (~ 0·5 lbf/in² (3·5 kPa)) from a longer distance (~ 100 μm). Pressure was applied continuously during the period indicated by the bottom bar; the hyperpolarizing pulses were 48 mV in amplitude. *C*, different concentrations of substance P (0·3 and 0·06 μM) were applied to one cell. The procedures were the same as in *A*, except that the holding potential was −79 mV, and the hyperpolarizing pulses were 48 mV in amplitude.

pressure using three 1 s duration pulses. Such an application of substance P produced a rapid decline in membrane conductance, which recovered slowly to its original value. The time course of recovery varied greatly from cell to cell; 90% recovery usually occurred between 25 and 110 s (mean = 67 s; $n = 16$) when 0·3 or 3 μM-substance P was applied. In addition to the conductance decrease, the substance P application induced a small (70–100 pA) inward-going change in holding current, which exhibited almost the same time course as the conductance change (Fig. 1).

This inward current is the counterpart under voltage clamp of the substance P-induced depolarization under constant current (Otsuka *et al.* 1982).

In Fig. 1*Ca* and *b*, two drug pipettes were used, and two different concentrations (300 and 60 nM) of substance P were applied successively onto the same neurone. As

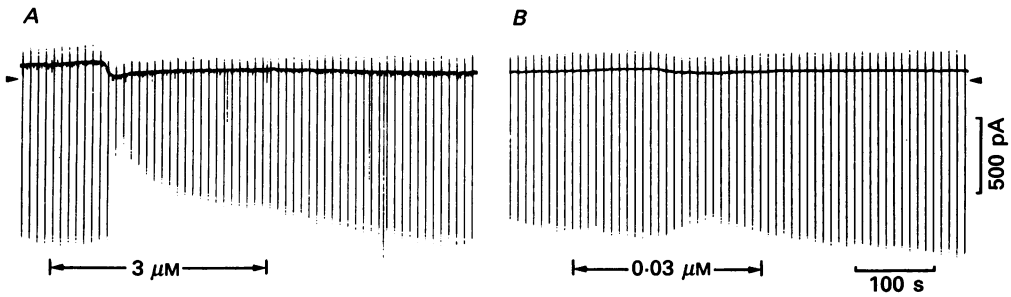

Fig. 2. Desensitization of the effect of substance P. *A*, desensitization produced by 3 μM-substance P. The whole-cell currents produced by intermittent voltage pulses were recorded: each pulse consisted of a square-wave depolarization (18 mV, 100 ms) followed by a 100 ms interval, and a square-wave hyperpolarization (48 mV, 100 ms). The holding potential was −74 mV. The superfusing solution was switched from normal 5 mM-K^+ Krebs (with 1 μM-tetrodotoxin) to the one containing substance P. After a dead time of about 1 min, the substance P effect began to appear, but the effect started to wane despite the continued presence of substance P. *B*, effect of 0·03 μM-substance P on another cell. The procedures and the conditions were the same as in *A*, except that the amplitude of hyperpolarizing square-wave pulse was 57 mV.

expected, 60 nM (Fig. 1*Cb*) produced less response than did 300 nM. When control Krebs solution, without substance P, was applied through the pipette, no change in conductance was normally obtained. However, on occasion, a small, apparent change in conductance was observed, as exemplified in Fig 1*Ab*. This artifact can be distinguished from the substance P-induced response by its quick time course as well as by the lack of concomitant inward current. Since the error produced by this artifact was small at the time when the current–voltage relationship was measured (5–15 s after substance P application), this error was ignored. The dose–response relationship was not systematically studied, but an estimate from experiments such as those in Fig. 1*Ca* and *b* indicated that 3 μM is well above the saturating concentration and the half-effective concentration of substance P was 40 nM or less.

Desensitization

The effect of substance P is known to desensitize (Dryer & Chiappinelli, 1985). In the experiments of Fig. 2, substance P was applied by exchanging the superfusing solutions. As shown in Fig. 2*A*, after about 1 min (representing the dead time) of switching from the control to the substance P-containing (3 μM) solution, the conductance started to decline, and an inward-going change in holding current appeared. However, despite the continuous presence of substance P, the conductance and inward current started to come back to their original levels with a half-time of approximately 50 s. This 'desensitization' occurred even with lower concentrations (30 or 300 nM) of substance P (Fig. 2*B*).

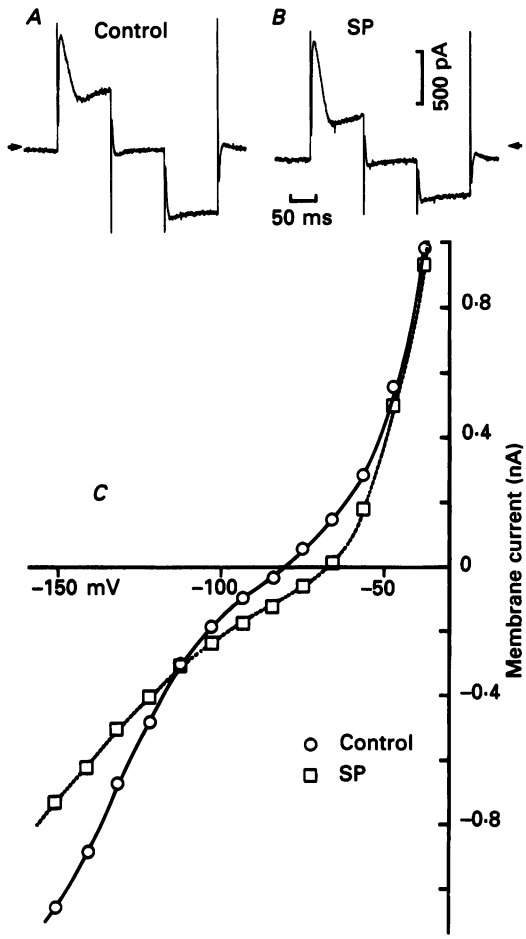

Fig. 3. *A*, whole-cell currents of a control neurone under voltage clamp. Square-wave voltage pulse sequence was: the cell was depolarized to −38 mV for 100 ms from holding potential of −84 mV; then after a 100 ms interval the cell was hyperpolarized to −132 mV for 100 ms. The depolarization produced a transient outward current (A-current), and the hyperpolarization produced an inward current (inward rectifying current). Arrows indicate the zero-current level. *B*, same as in *A*, except that the record was made while substance P (SP) effect was near its peak. A few extraneous noises (short spikes) were retouched. *C*, current–voltage relation in control (○) and during the action of substance P (SP, 3 μM; □). Same neurone as in *A* and *B*. The external solution was 2·5 mM-K^+ Krebs with tetrodotoxin. The currents were plotted at 10 ms of applying voltage steps.

Substance P-sensitive current

The properties of the conductance affected by substance P were examined by the following procedures. First, the current–voltage relationship was measured for a cell in 2·5 mM-K^+ external solution (Fig. 3). The usual sequence of voltage commands was to apply a square-wave depolarization of 100 ms duration followed by a 100 ms pause and then a square-wave hyperpolarization of 100 ms (Fig. 3*A*). This sequence was repeated once every second using various amplitudes of polarization. Then, substance

P was applied by ejection (1 s duration ejection, 3 times in rapid succession). A few seconds after the end of substance P application, while the substance P effect was at its height, the current–voltage relation was again measured (Fig. 3*B*).

In the control neurone, when a small hyperpolarizing voltage pulse was applied, the time course of the inward current appeared almost square in shape. However,

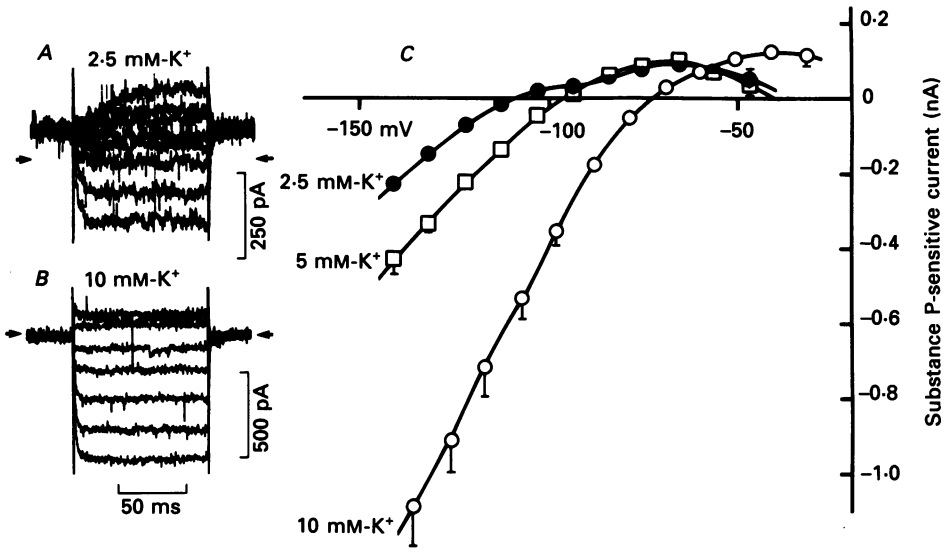

Fig. 4. *A*, substance P-sensitive currents in 2·5 mM-K^+ solution. Holding potential was −84 mV. Potential level for each record was (from the uppermost): −56, −66, −75, −94, −103, −113, −122 and −132 mV. The arrows indicate the zero-current (substance P-sensitive current) level. The records were made by subtracting record during substance P action (such as in Fig. 3*B*) from control record (such as in Fig. 3*A*); the record was then shifted and superimposed. *B*, substance P-sensitive currents from another neurone in 10 mM-K^+ solution. The holding potential was −69 mV; the potential level for each record was (from uppermost): −41, −51, −60, −79, −88, −98, −107 and −117 mV. There is a crossing in records at −41 and −51 mV. *C*, mean values of substance P-sensitive currents plotted against potential for three $[K^+]_o$ solutions. The sample numbers were: $n = 11$ in 2·5 mM-K^+, $n = 9$ in 5 mM-K^+ (except that $n = 6$ at −141 mV point), $n = 13$ in 10 mM-K^+. Vertical bars, 1 S.E.M. In early experiments the substance P-sensitive currents were measured by the digitizer–computer combinations by plotting currents at 10–15 ms of the beginning of voltage step. In later experiments, comprising about one-half of the data, the substance P-sensitive currents (such as in *A* or *B*) were computed, and then the current–voltage relation was measured by computer. (The average diameter of the soma was 27 μm.)

when larger hyperpolarizations were applied bringing the potential more negative than −120 mV, the current, after reaching its peak, started to decline slightly with a slow time course (Fig. 3*A*). This decline could reflect the time-dependent inactivation of the inward current. When large depolarizations (say, more depolarized than −50 mV) were applied, the current reached a large initial peak and then declined quickly, suggesting activation of A-current (Fig. 3*A*).

In Fig. 3*C*, current–voltage relations were plotted before application of substance P (○) and during the effect of substance P (□). The measurements of current

amplitude were made 10 ms after the beginning of the voltage step. Current–voltage relations in control conditions as well as during the substance P effect show rectification in the inward direction, larger hyperpolarizations producing larger inward currents.

Further insight into the substance P effect can be obtained by subtracting the current during the action of substance P from the control current. This current will be referred to as the 'substance P-sensitive current,' since it reflects the current through ionic channels that are modulated by substance P. Figure 4*A* and *B* shows examples of the substance P-sensitive current. The currents are almost square-shaped over the range of −60 to −130 mV, except for an initial delay of about 10 ms. In some cells, there was a small time-dependent increase in the current in addition to the initial delay, but we did not observe a time-dependent decrease (inactivation) during the 100 ms duration. This time course is very similar to the acetylcholine-induced current in the heart muscle (Sakmann, Noma & Trautwein, 1983) or to the somatostatin-induced current in brain neurones (Inoue, Nakajima & Nakajima, 1988).

In Fig. 4*C* mean values of the substance P-sensitive current (measured at 10–15 ms of the beginning of the voltage step) were plotted against the voltage in three external K^+ concentrations (2·5, 5 and 10 mM). Each curve shows an obvious rectification in the inward direction, and reveals a distinct reversal potential, at which the substance P-sensitive current changes its direction. As the external K^+ concentration increased this reversal potential shifted to the depolarizing direction, and the slope of the current–voltage relation increased.

In Fig. 5, the reversal potentials of the substance P-sensitive currents were plotted against external K^+ concentration. The continuous line, which is the regression line of the data, indicates that a 10-fold change in $[K^+]_o$ produced a 60·7 mV change in the reversal potential. The dashed line represents the theoretical values of K^+ equilibrium potential at 30 °C assuming that $[K^+]_i$ is 130 mM (the potassium concentration of the intrapipette solution). The slope of the theoretical line (60·2) coincides well with the slope of the regression line; however, the regression line is situated at more negative points than the theoretical line by 6 mV. A small part of this discrepancy would be due to the errors produced by the series resistance. We assumed that the average series resistance was 5·8 MΩ (twice the value of electrode resistance) and calculated the errors from the averaged current–voltage relation of the neurone sample used for Fig. 5. The data showed that the real reversal potential would have been more positive on the average by 1·2 mV. Thus the discrepancy between the data and the prediction is 5 mV.

The conclusion of the results of Fig. 5 is that the substance P-sensitive conductance is primarily a potassium conductance, although the reversal potentials do not perfectly coincide with the theoretical prediction (see Discussion). Further the current–voltage relation is very similar to that of the inward rectifying channels in skeletal muscle fibres or oocytes (Adrian & Freygang, 1962*a*; Hagiwara & Takahashi, 1974).

(When the neurone was depolarized beyond −50 mV, conductances other than the inwardly rectifying channels start to occur. Figure 3*A* and *B* shows the presence of a rapidly inactivating current, probably A-current, as well as an outward current

activated with a delay, possibly delayed K^+ current. We did not do a systematic investigation of substance P effects on these currents, but their amplitudes appeared not to be changed by substance P.)

Substance P-sensitive conductance

The current voltage relationships of Fig. 4 allow us to calculate the chord conductance of substance P-sensitive channels. The chord conductance is given by

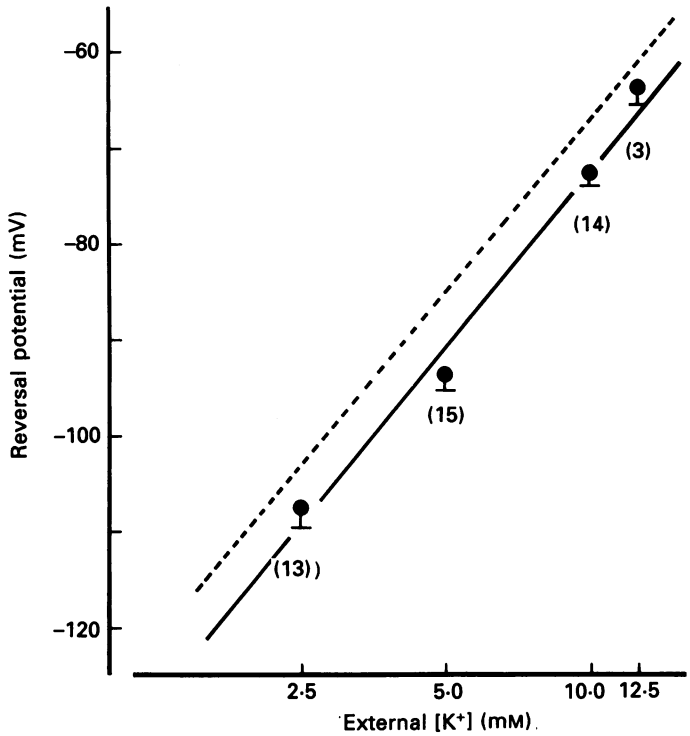

Fig. 5. Reversal potentials of substance P-sensitive current plotted against external K^+ concentrations. The number of cells is indicated in parentheses, and the vertical bars are 1 S.E.M. The continuous line is the regression line through the data: $V_{rev} = 60{\cdot}7 \log ([K^+]_o/157)$. The dashed line is the potassium equilibrium potential at 30 °C on the assumption that K^+ concentration inside the cell is the same as that of patch solution; namely $E_K = 60{\cdot}2 \log ([K^+]_o/130)$.

dividing current by electromotive force $(V-V_{rev})$, in which V is the membrane potential and V_{rev} is the reversal potential. In Fig. 6, the mean values of the substance P-sensitive conductances were calculated from the data of Fig. 4. Experimental values were fitted by a Boltzmann equation, namely:

$$g = g_{max}/(1+\exp[(V-V')/k]), \quad (1)$$

where g is the chord conductance, g_{max} represents the maximum conductance at each K^+ concentration, and V' and k are parameters determining the half-activation voltage and the steepness, respectively. Table 1 summarizes the values for these parameters. The half-activation voltage V' shifted to the positive direction as $[K^+]_o$

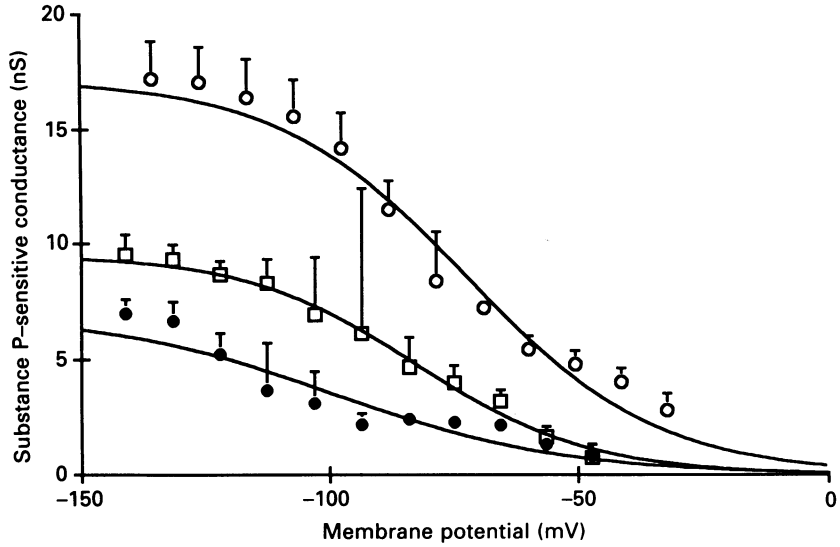

Fig. 6. Chord conductance of the mean substance P-sensitive current plotted against membrane potential. Data were taken from Fig. 4. ●, 2·5 mM-K^+; □, 5 mM-K^+; ○, 10 mM-K^+. Vertical bar = 1 S.E.M. The data are fitted by the Boltzmann expression: $g = g_{max}/[1+\exp\{(V-V')/k\}]$. For the values of the parameters and the procedure of curve fitting see Table 1.

TABLE 1. Parameters of the Boltzmann equation fitted to the Substance P-sensitive conductance

	g_{max} (nS)	k (mV)	V' (mV)	V_K (mV)	$(V'-V_K)$ (mV)
2·5 mM-K^+	7·0	23·3	−99	−103	4
	(8·9)	(20·4)	(−102)		(1)
5 mM-K^+	9·5	17·5	−83	−85	2
	(12·1)	(14·4)	(−85)		(1)
10 mM-K^+	17·2	19·3	−73	−67	−6
	(23·1)	(14·8)	(−76)		(−9)

The values in parentheses were calculated after the error due to the series resistance was corrected. The curve fitting was performed by the least-squares method using the Simplex algorithm. When the three parameters were varied freely, the curve fitting produced somewhat unrealistic parameter values. Therefore, the conductance value at the most hyperpolarized potential was assigned to g_{max} for each data set.

is increased, suggesting that the activation curve is determined at least approximately by the driving force on potassium ions rather than on voltage only. The steepness factor k is 14·5–20 mV. The value of g_{max} increased with $[K^+]_o$: a four-fold increase in $[K^+]_o$ produced a 2·5-fold increase in g_{max}, i.e. g_{max} is proportional to $([K^+]_o)^{0.65}$. If the series resistance is compensated, g_{max} is proportional to $([K^+]_o)^{0.69}$. In six cases the same neurone was used to measure the substance P-sensitive conductance in two different K^+ concentrations (2·5 mM then 10 mM or in the reverse order). In this sample, g_{max} was proportional to $([K^+]_o)^{0.63}$. The results of this section again indicate that the substance P-sensitive channels have properties similar to

those of the inward rectification channels described in skeletal muscles or oocytes (Hagiwara & Takahashi, 1974; Leech & Stanfield, 1981).

Effects of Cs^+

The inward rectification in skeletal muscles or oocytes is inhibited by Cs^+ at relatively low concentrations. This inhibition is probably due to block of channels,

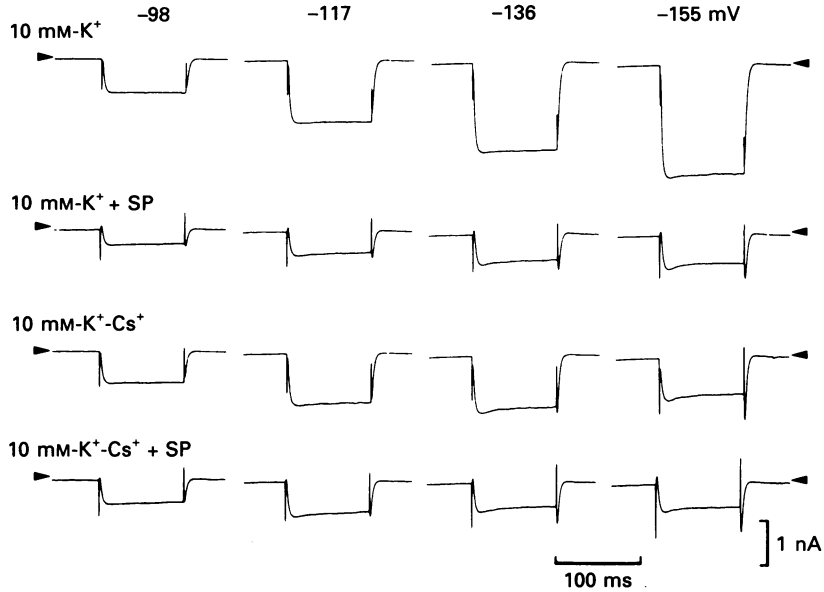

Fig. 7. Membrane current elicited by square-wave hyperpolarizing voltage pulses in 10 mM-K^+ Krebs solution, and 0·1 mM-Cs^+-containing solution before and during substance P (SP; 3 μM) action. Holding potential, −69 mV. The arrow-heads indicate the zero current levels.

and it occurs in a voltage-dependent manner (Hagiwara, Miyazaki & Rosenthal, 1976; Gay & Stanfield, 1977). We have examined the effect of Cs^+ (0·1 mM) on the substance P-sensitive current as well as on control neurones (i.e. before application of substance P). The experiment of Fig. 7 was carried out using the following sequence. First, the current–voltage relationship was measured in 10 mM-K^+ solution, then substance P was applied and the current–voltage relationship was again determined while the substance P effect was still near its height. Now the superfusing solution was switched to 10 mM-K^+ solution with 0·1 mM-Cs^+ added, and the current–voltage relationship was again measured. Finally, substance P was ejected with 0·1 mM-Cs^+, and the current–voltage relationship measured during the substance P effect. From these data we determined the effect of Cs^+ on the control neurone and the substance P-sensitive current (Fig. 8).

Figure 8*A* shows effects of Cs^+ (0·1 mM) on the control current. Cs^+ did not have much influence on the membrane currents between −50 and −110 mV. However, the effect became evident as the potential was negative to −110 mV. When the voltage was negative to −130 mV, the Cs^+ inhibition became pronounced and a

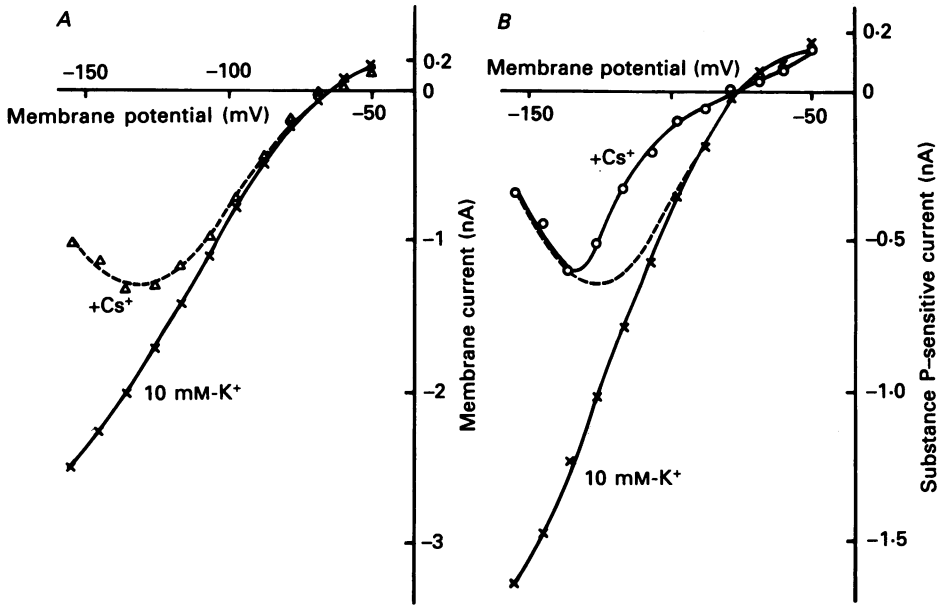

Fig. 8. *A*, current–voltage relationship of the membrane measured in 10 mM-K^+ solution (×) and then in 0·1 mM-Cs^+-containing 10 mM-K^+ solution (△). The current magnitudes were measured at the initial peak (~ 15 ms after the start of the voltage step). The dashed line was calculated from the data in 10 mM-K^+ using eqns (2) and (3) of the text with $\mu = 1{\cdot}33$. *B*, current –voltage relationship of the substance P-sensitive current in 10 mM-K^+ solution (×) and in 0·1 mM Cs^+-containing 10 mM-K^+ solution (○). The dashed line was calculated from the data in 10 mM-K^+ solution using eqns (2) and (3) with $\mu = 1{\cdot}70$.

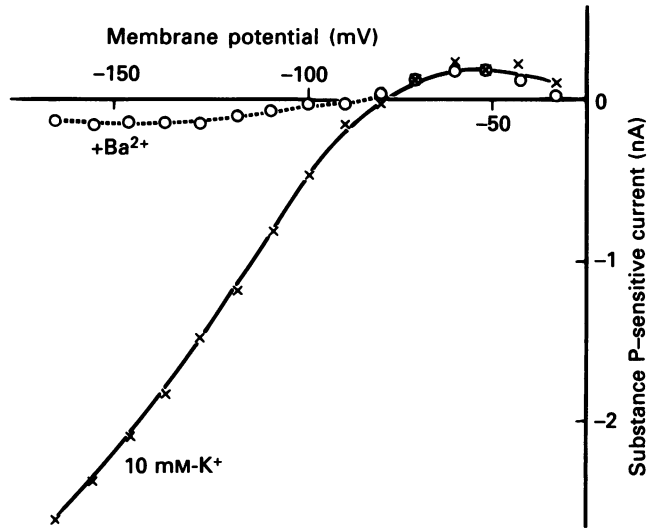

Fig. 9. Current–voltage relation of substance P-sensitive current in 10 mM-K^+ solution (×) and 0·05 mM-Ba^{2+}-containing 10 mM-K^+ solution (○). The current magnitudes were measured at the end of 100 ms voltage pulse. The dotted line was calculated from the data of 10 mM-K^+ solution using eqns (2) and (3) with $\mu = 0{\cdot}11$.

negative conductance region appeared. This behaviour of Cs^+ inhibition can be predicted from the theory proposed by Hagiwara *et al.* (1976) or by Standen & Stanfield (1978) in which it was assumed that Cs^+ binds in a voltage-dependent manner to a site within the inward rectifier channels, and this binding leads to channel blockage. In this model the inhibition of the inward current is expressed by a parameter y, which represents the ratio of the inward current in the presence of Cs^+ to that in the absence of Cs^+, where

$$y = 1/(1+[Cs^+]_o/K_d), \tag{2}$$

In which K_d is the dissociation constant for binding of Cs^+ to its blocking site. The dissociation constant is voltage dependent, so that,

$$K_d = K_o \exp(\mu FV/RT), \tag{3}$$

where K_o is the dissociation constant at zero potential, μ, the equivalent valency, represents the membrane potential dependence of Cs^+ binding, and corresponds to δz, namely electrical distance multiplied by valency, and F, R and T have their usual meanings. The predicted values in the presence of Cs^+ were indicated by the dashed line of Fig. 8*A*, which was calculated from the data without Cs^+ assuming that $\mu = 1{\cdot}33$ and $K_o = 166$ mM. These latter values were obtained by linear regression after plotting $\ln[y/(1-y)]$ against voltage. The calculated curve fits the experimental data very well. The average value of equivalent valency μ for five experiments was $1{\cdot}34 \pm 0{\cdot}35$ (mean $\pm$ S.E.M.).

The substance P-sensitive currents, in the absence and in the presence of Cs^+, are shown in Fig. 8*B*. Clearly Cs^+ at 0·1 mM inhibited substance P-sensitive current substantially in a voltage-dependent manner. The dashed line of Fig. 8*B* was calculated based on the data without Cs^+ assuming μ to be 1·7 and K_o to be 600 mM. These parameters were determined using the data over voltages negative to -136 mV. The mean value of μ determined from data at voltages negative to 136 mV was $1{\cdot}9 \pm 0{\cdot}24$ ($n = 4$).

Effects of Ba^{2+}

Barium has also been shown to inhibit inward rectification in other cells (Hagiwara, 1983; Standen & Stanfield, 1978). Interestingly, Ba^{2+} does not inhibit another type of channel called I_h (or I_f) (Brown & DiFrancesco, 1980; Yanagihara & Irisawa, 1980). I_h is caused by permeability increase to Na^+ and K^+ activated by hyperpolarizations in sinoatrial node cells. A similar conductance was described in hippocampal neurones and called I_Q (Halliwell & Adams, 1982).

We have tested the effect of Ba^{2+} (0·05 mM) on our cultured neurones (Fig. 9). In contrast with that of Cs^+, the effect of Ba^{2+} on the substance P-sensitive current was not strongly voltage dependent. In Fig. 9 the dotted line was calculated according to an appropriate form of eqn (2) and eqn (3) using 0·11 as the value of equivalent valency (μ). In another cell the value of μ was 0·38, and in two other cells there was no apparent voltage dependence in the effect of Ba^{2+}. (Figure 9 shows that Ba^{2+} is not effective on the depolarizing side. Since the barium effect is not very voltage dependent, it ought to show its effect on the depolarizing side. Obviously the simple theory is not applicable to the wide range of voltage.)

Inactivation of inward rectification and the effect of substance P

When the capacitative transient was neutralized as fully as possible, a moderate hyperpolarizing voltage step produced an inward current that was almost square, except for the initial 10 ms (Fig. 7, −98 mV). However, larger hyperpolarizations

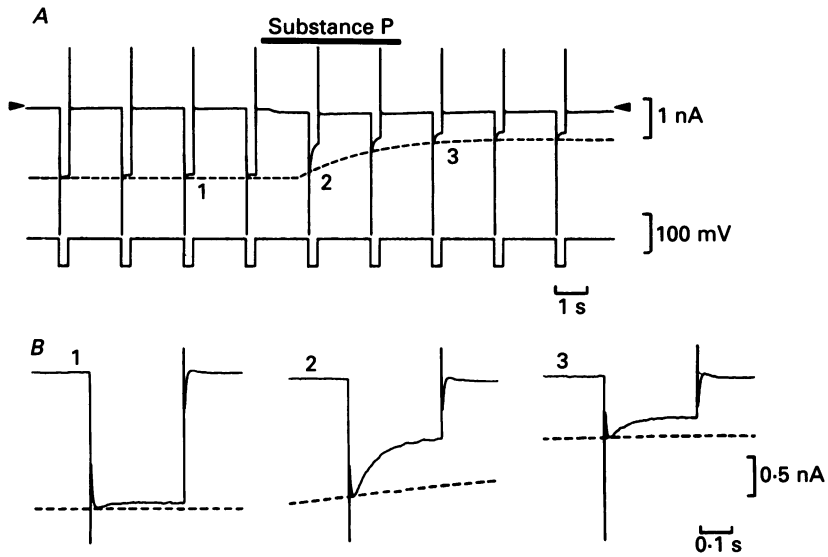

Fig. 10. *A*, enhancement of time-dependent inactivation during the action of substance P in 5 mM-K^+ solution. Intermittent hyperpolarizing voltage pulses (67 mV amplitude) from a holding potential of −79 mV (lower trace record, voltage) were applied and the resulting currents were recorded (upper trace). The horizontal bar indicates the timing of applying substance P (3 μM) by pressure ejection. Arrow-heads indicate the zero current level. The peaks of the inward currents were connected with a single exponential curve (dashed line) with a time constant of 2·0 s. *B*, current records at expanded time scale, record 1, 2, 3 corresponding to the record 1, 2, 3 in *A*. The dashed lines in *B* correspond to the dashed line in *A*.

produced an inward current that declined slowly and incompletely (Fig. 3; Fig. 7, −155 mV). This decline is probably an inactivation with time of the channels that produce inward rectification. Although there was considerable cell-to-cell variation, the inactivation process was not very prominent, typically producing a decline of no more than a few per cent in 100 ms.

Substance P appeared to transiently enhance this time-dependent inactivation. This enhancement is illustrated in the experiment of Fig. 10, where hyperpolarizing voltage steps were applied intermittently. Before application of substance P, little inactivation was seen in the inward current (Fig. 10*A*; the record marked 1 is shown in Fig. 10*B*1 on an expanded time scale). Although an accurate evaluation of the extent of the inactivation is difficult because of the presence of capacitative current associated with the charging of neurites, the decline of the inward current was not more than 5% of the peak amplitude. As the application of substance P was begun, the inward current showed a more prominent decline (record 2 of Fig. 10*A* and *B*).

In evaluating the time course and extent of inactivation when substance P is first applied, it is necessary to take into account the fact that the records were taken while the inward rectification is being diminished by substance P. The time course of the gradual onset of the effect of the peptide is shown by the dashed line in Fig. 10*A*,

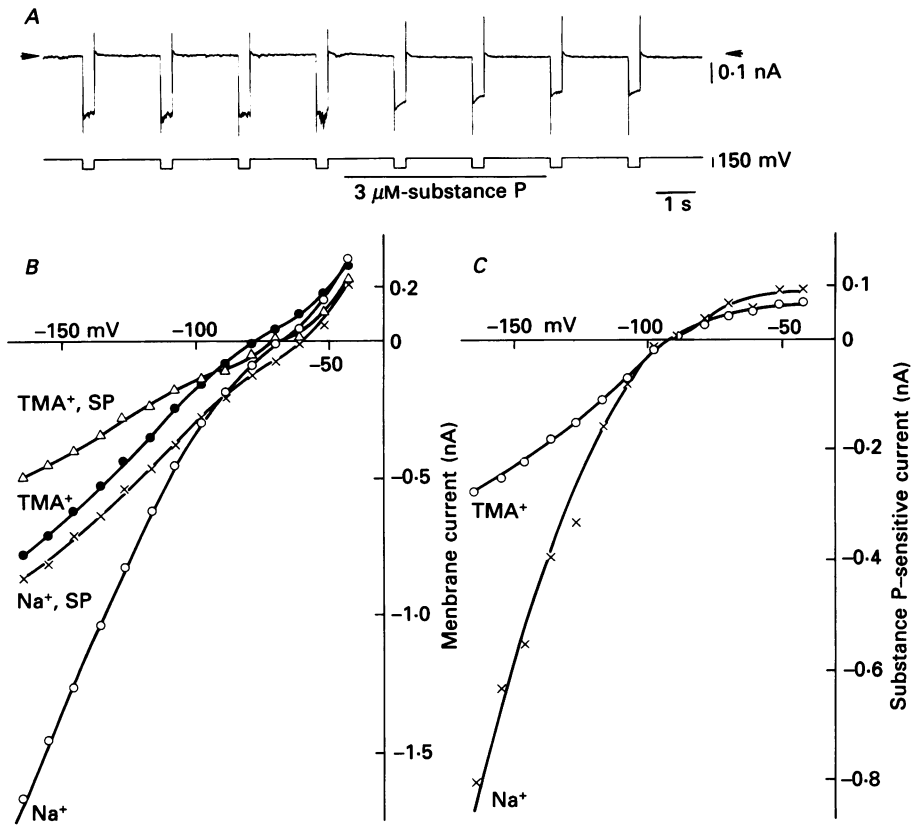

Fig. 11. *A*, time course of the action of substance P in Na^+-free, tetramethylammonium ion solution. Upper trace current; lower trace, voltage. Holding potential, −79 mV. Arrows indicate the zero current level. The horizontal line indicates the timing of substance P (3 μM) application. *B*, current–voltage relation in Na^+-Krebs solution, and in TMA^+-solution before and during the action of substance P (SP) in another cell. *C*, substance P-sensitive current in Na^+-Krebs and in TMA^+ solutions. $[K^+]_o$ was always 5 mM.

which encloses the envelope of successive current peaks. This envelope is also indicated in the expanded records of Fig. 10*B*. When this gradual increase in substance P effect is taken into account, the degree of inactivation was 45% in record 2. When the substance P effect developed fully, the inactivation of the inward current became less prominent, while still remaining more obvious than in control; records 3 of Fig. 10*A* and *B* shows that the degree of inactivation was 27%. The current after full development of substance P effect would reflect the activity of the remaining channels (primarily substance P-resistent channels). Thus, the substance P-resistant channels show inactivation, whereas the substance P-sensitive current

does not decline, as explained previously (Fig. 4). The implication of these phenomena will be discussed later.

Although the degree of inactivation appeared to be enhanced by substance P, there was no statistically significant change in the time constant of inactivation, the mean values being 98 ± 8 ms (mean $\pm$ S.E.M., $n = 7$) in control and 131 ± 17 ms ($n = 7$) in the presence of substance P at voltage between -127 and -165 mV.

Inactivation of inward rectification and external Na^+

In skeletal muscle and oocytes, inactivation of inward rectification with time under hyperpolarization is caused by ionic blockage of ion channels by external Na^+ (Ohmori, 1978; Standen & Stanfield, 1979). On the other hand inactivation of inward rectification is, in cardiac muscle, associated with an intrinsic voltage-dependent gating of channels (Sakmann & Trube, 1984). Segal & Barker (1984) have reported that the presence of external Na^+ is essential for the activation of inward current under hyperpolarization in hippocampal neurones. Thus, we have examined the effects of removal of Na^+ on the substance P-sensitive current.

Current–voltage relations are plotted in Fig. 11 in normal Krebs solution and then in a Na^+-free external solution, made by replacing Na^+ with tetramethylammonium ions (TMA^+). Although in some cells replacement of Na^+ by TMA^+ reduced both control inward currents and the substance P-sensitive current, the effect was relatively small (and smaller on average than that seen in the neurone illustrated in Fig. 11). For the control current, the decrease at -155 mV was by 17 ± 12% ($n = 5$), while the reduction in substance P-sensitive current was 18 ± 19% ($n = 5$) at the same voltage. Further the time-dependent inactivation of the inward current was still present and was still enhanced by substance P (Fig. 11*A*) indicating that the process is not caused by a Na^+-dependent blockage of ion channels.

The reversal potential of currents was not significantly altered by Na^+ replacement (although a slight shift is apparent in Fig. 11). In 2·5 mM-K^+, the reversal potential of the substance P-sensitive current was $-93{\cdot}4 \pm 1{\cdot}7$ mV ($n = 15$) in the presence of Na^+ and $-90{\cdot}3 \pm 3{\cdot}4$ mV ($n = 7$) in its absence. The conductance modified by the tachykinin is therefore not that associated with I_Q, a current carried by both K^+ and Na^+ (Halliwell & Adams, 1982).

Effects of internal Na^+

In starfish oocytes (though apparently not in skeletal muscle), internal Na^+ is required for the presence of inward rectification (Hagiwara & Yoshii, 1979). In our experiments on neurones, removal of Na^+ from the patch pipette (and therefore from the intracellular solution) and its replacement with K^+ altered neither inward rectification in control, nor the substance P-sensitive current (fifteen cells examined).

Effects of sodium nitroprusside

Konishi & Okamoto (1985) proposed cyclic GMP as a second messenger that mediates the effect of substance P in sympathetic ganglion cells. This conclusion was based partly on the result that sodium nitroprusside, an activator of guanylate cyclase, produces a slow depolarization.

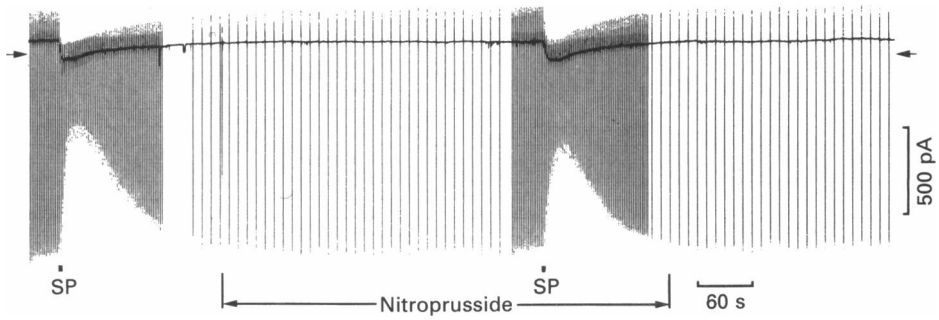

Fig. 12. Effects of sodium nitroprusside on the response to substance P. The whole-cell clamp technique was used. Currents produced by intermittent voltage pulses were recorded: each pulse consisted of a square-wave depolarization (18 mV, 100 ms) followed by a 100 ms interval and a square-wave hyperpolarization (48 mV, 100 ms). The holding potential was −74 mV. First, substance P application by pressure ejection (SP; 0·3 μM) produced a substantial decrease of conductance concomitant with an inward current. The superfusing solution was 5 mM-K^+ Krebs solution. Then the superfusing solution was switched to a Krebs solution containing sodium nitroprusside (20 μM). This did not result in any change of membrane conductance or holding current. While the culture was superfused with sodium nitroprusside, another application of substance P (0·3 μM) again produced almost the same effect as the first application. The dead time of the superfusion system was about 1 min.

nitroprusside. This manoeuvre failed to produce a change in either the membrane conductance or the level of holding current. Further, in the presence of sodium nitroprusside, when substance P was applied, it still produced a response very similar to that obtained in normal Krebs solution. Essentially the same results was obtained in another cell.

We also examined the effect of sodium nitroprusside on our neurones using conventional intracellular recording with an impaling microelectrode. Out of twelve cells that showed a clear depolarization by substance P application, only in three was a long-lasting depolarization produced by sodium nitroprusside, there being no detectable effect in the remainder.

DISCUSSION

Substance P-sensitive current

It has recently been discovered that several neurotransmitters or neuromodulators exert their effects by modulating the inward rectifying channels in invertebrate (Benson & Levitan, 1983) and vertebrate neurones (Stanfield *et al.* 1985; Mihara, North & Surprenant, 1987; Inoue *et al.* 1988). However, the properties of the inward rectifying channels that may be modulated have not been investigated thoroughly. Since the discovery of the inward rectifying channels by Katz (1949), the channels

have been the subject of intensive biophysical studies and their properties are well documented in skeletal muscles and oocytes (Stanfield, Standen, Leech & Ashcroft, 1981; Hagiwara, 1983). The main objective of the present investigation was to make a detailed analysis of biophysical properties of the channels that are modulated by substance P. This is important since the mere presence of inwardly rectifying current–voltage relations does not necessary reveal the identity of the channels.

The present results indicate that the substance P-sensitive current is carried by K^+ through the inward rectifier channels. The reasons are the following.

(1) The reversal potential of the substance P-sensitive current was dependent on external $[K^+]$. In the range 2·5–12·5 mM, the reversal potential changed by 60 mV for a ten-fold change $[K^+]_o$, a change almost identical with that predicted by the Nernst equation (Fig. 5). This agreement with theory appears to indicate that the major ions through the channels are potassium. The measured values of reversal potential were, however, 5 mV more negative than anticipated. Possible reasons for this discrepancy include errors in the estimated value of series resistance, the possibility that $[K^+]_i$ was higher than that in the recording patch pipette, and a possible contribution of the current from dendritic membrane located away from the recording site.

(2) The shape of the current–voltage relation for the substance P-sensitive current showed typical inward rectification, and the associated chord conductance changed not only with membrane potential, but also with $[K^+]_o$ (Fig. 6). This apparent dependence on the driving force on K^+ has been demonstrated for the inward rectifier of skeletal muscle fibres and starfish egg cells (Hodgkin & Horowicz, 1959; Hagiwara & Takahashi, 1974; Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981). In the measurements of Hagiwara & Takahashi (1974) the value of the steepness factor k, used to fit a Boltzmann expression to the relation between chord conductance and membrane potential (see eqn (1)) was 6·7 mV. In the present experiments on mammalian neurones, this steepness factor had a value of 14·5–20 mV. Such a value is consistent with a charged element controlling the gating of these inward rectifier channels having equivalent valency of between 1·3 and 1·8.

(3) In addition to the apparent dependence of the conductance on the driving force on K^+, the value of the maximum chord conductance also depended on $[K^+]_o$. In starfish egg (Hagiwara & Takahashi, 1974) where a similar property is found, this maximum conductance increases with $([K^+]_o)^{0.5}$. In our experiments on neurones, the substance P-sensitive conductance increases over all our experiments with $([K^+]_o)^{0.7}$. In experiments where measurements were made in the same cell in more than one value of $[K^+]_o$, the conductance increased with $([K^+]_o)^{0.63}$.

(4) Low concentrations of Cs^+ (0·1 mM) and Ba^{2+} (0·05 mM) blocked the substance P-sensitive current in a voltage-dependent manner (Figs 8 and 9), as reported also for inward rectifiers of egg cells (Hagiwara *et al.* 1976) and skeletal muscle fibres (Gay & Stanfield, 1977; Standen & Stanfield, 1978). In neurones, as in starfish egg and skeletal muscle, the voltage-dependent block of Cs^+ could be fitted by an expression that assumed one-to-one binding of Cs^+ to a site causing channel blockage, with the dissociation constant being voltage-dependent (Fig. 8*A*). The attempt to fit the Cs^+ blockage of substance P-sensitive current under substantial hyperpolarization using the assumptions given above gave an equivalent valency of Cs^+ as a blocking ion of 1·9 (the value of μ in eqn (3) of text), a value similar to that fits block by Cs^+ in egg

cell (1·4–1·5, Hagiwara *et al.* 1976), and in skeletal muscle (1·4; Gay & Stanfield, 1977; Gay, 1979). As Hille & Schwarz (1978) have described, an equivalent valency greater than one for Cs^+ indicates block of a multi-ion pore.

The blockage by Ba^{2+} which we have found for the substance P-sensitive current is less steep in its voltage dependence than that found for inward rectifiers of egg cells or skeletal muscle. Here the equivalent valency of Ba^{2+} as a blocking ion is 0–0·38, while values of around 1·4 were found by Standen & Stanfield (1978) for skeletal muscle. The fact that Ba^{2+} acts as a blocker probably rules out the involvement of I_Q, described in hippocampal neurones by Halliwell & Adams (1982).

In addition to blocks by Cs^+ and Ba^{2+}, the substance P-sensitive current was blocked by extracellular Rb^+ and by tetraethylammonium ions, as we have previously reported (Stanfield *et al.* 1985).

All these results suggest that the substance P-sensitive current is through inwardly rectifying K^+ channels whose basic properties are similar to those found in other cells.

Inactivation of inward rectification

The process underlying the decay under hyperpolarization of inward currents through inward rectifier channels has been analysed by a number of authors (Adrian & Freygang, 1962*b*; Adrian, Chandler & Hodgkin, 1970; Stanfield, 1970; Ohmori, 1978; Standen & Stanfield, 1979; Kameyama, Kiyosue & Soejima, 1983; Sakmann & Trube, 1984). In skeletal muscle the decay was attributed to depletion of K^+ from the lumen of the transverse tubular system and to blockage by external Na^+ (Adrian & Freygang, 1962*b*; Adrian *et al.* 1970; Standen & Stanfield, 1979), and Na^+ block was also shown to cause inactivation in the inward rectifier of egg cells (Ohmori, 1978). However, in cardiac cells the inward rectifier inactivates in the absence of external Na^+ (Kameyama *et al.* 1983; Sakmann & Trube, 1984), and the inactivation process is due to intrinsic voltage-dependent gating. Our own results show that in cultured cholinergic neurones from nucleus basalis, the inward rectifier in control neurones continues to inactivate in the absence of external Na^+ and that in this respect the K^+ channels of neurones are similar to those of heart muscle.

Our results also suggest that substance P appears to enhance this inactivation process. The action appeared to be to alter the degree of inactivation rather than its time course and the enhancement was greater during the first few seconds of application of substance P than subsequently. However, computer subtraction of the substance P-sensitive current suggests that, in the steady state of substance P action, there is no inactivation of the current blocked by the tachykinin. These results are consistent with the hypothesis that there are two kinds of K^+ channels contributing to the resting K^+ permeability. One may be modulated by substance P and shows little or no sign of time-dependent inactivation; the other type, which is not modulated by substance P, will consequently remain open after the peptide has reduced the contribution of the first type, and shows time-dependent inactivation. The same hypothesis was proposed to explain the effect of somatostatin on locus coeruleus neurones (Inoue *et al.* 1988). Also, the existence of two kinds of K^+ channels, one that is modulated by acetylcholine, the other that is not modulated, was described in heart muscle (Sakmann *et al.* 1983). The transient enhancement of

inactivation during the substance P application can be explained by incorporating an idea that a process analogous to open channel blocking is involved in the substance P modulation of the K^+ channels.

However, we are unable, in the absence of confirmatory evidence from single-channel recording, to rule out entirely the alternative hypothesis that there is a single class of potassium channels whose gating properties are modulated by substance P.

Our result also show that, in contrast to the situation described in hippocampal neurones by Segal & Barker (1984), inward rectification in neurones of the nucleus basalis is not abolished by removal of external Na^+. Further, unlike the inward rectifier of egg cells (Hagiwara & Yoshii, 1979), the conductance is not abolished by removal of internal Na^+.

Second messenger mechanisms and the action of substance P

It is likely that the action of substance P on inward rectification is mediated through some as yet unidentified second messenger mechanism, though apparently this effect is mediated through a pertussis toxin-resistant G-protein (Nakajima, Nakajima & Inoue, 1988). One possibility examined here is that, like the effect of substance P in sympathetic ganglion cells (Konishi & Okamoto, 1985), the action is further mediated through cyclic GMP, presumably through a cyclic GMP-dependent protein kinase. Sodium nitroprusside, an activator of guanylate cyclase, failed in our whole-cell clamp experiments to mimic the actions of substance P. Since the primary action of sodium nitroprusside might be on cytosolic guanylate cyclase, and since this enzyme is likely to be washed out during whole-cell recording, we also investigated the actions of sodium nitroprusside in intact cells with conventional impaling microelectrodes. Here, the compound did occasionally produce a slow depolarization. However, since this response was found in only a few cells, and since the response to substance P was consistently found in the conditions of whole-cell recording, it is unlikely that the presently described action of the tachykinin depends on the production of cyclic GMP.

This research was supported by NIH grant AG06093 and an Alzheimer's Disease and Related Disorders Association Grant and by a travel grant to P.R.S. from the Wellcome Trust.

REFERENCES

ADAMS, P. R., BROWN, D. A. & JONES, S. W. (1983). Substance P inhibits the M-current in bullfrog sympathetic neurones. *British Journal of Pharmacology* **79**, 330–333.

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1970). Slow changes in potassium permeability in skeletal muscle. *Journal of Physiology* **208**, 645–668.

ADRIAN, R. H. & FREYGANG, W. H. (1962*a*). Potassium conductance of frog muscle membrane under controlled voltage. *Journal of Physiology* **163**, 104–114.

ADRIAN, R. H. & FREYGANG, W. H. (1962*b*). The potassium and chloride conductance of frog muscle membrane. *Journal of Physiology* **163**, 61–103.

BEACH, T. G., TAGO, H. & MCGEER, E. G. (1987). Light microscope evidence for a substance P-containing innervation of the human nucleus basalis of Meynert. *Brain Research* **408**, 251–257.

BENSON, J. A. & LEVITAN, I. B. (1983). Serotonin increases an anomalously rectifying K^+ current in the *Aplysia* neuron R15. *Proceedings of the National Acadamy of Sciences of the USA* **80**, 3522–3525.

BOLAM, J. P., INGHAM, C. A., IZZO, P. N., LEVEY, A. I., RYE, D. B., SMITH, A. D. & WAINER, B. H. (1986). Substance P-containing terminals in synaptic contact with cholinergic neurons in the neostriatum and basal forebrain: a double immunocytochemical study in the rat. *Brain Research* **397**, 279–289.

BROWN, H. & DIFRANCESCO, D. (1980). Voltage-clamp investigations of membrane currents underlying pace-maker activity in rabbit sino-atrial node. *Journal of Physiology* **308**, 331–351.

BROWNSTEIN, M. J., MORZ, E. A., KIZER, J. S., PALKOVITS, M. & LEEMAN, S. E. (1976). Regional distribution of substance P in the brain of the rat. *Brain Research* **116**, 299–305.

COYLE, J. T., PRICE, D. L. & DELONG, M. R. (1983). Alzheimer's disease, a disorder of cortical cholinergic innervation. *Science* **219**, 1184–1190.

DRYER, S. E. & CHIAPPINELLI, V. A. (1985). Properties of choroid and ciliary neurons in the avian ciliary ganglion and evidence for substance P as a neurotransmitter. *Journal of Neuroscience* **5**, 2654–2661.

GAY, L. A. (1979). Potassium conductance of frog skeletal muscle. Ph.D. Thesis, University of Leicester.

GAY, L. A. & STANFIELD, P. R. (1977). Cs^+ causes a voltage-dependent block of inward K currents in resting skeletal muscle fibres. *Nature* **267**, 169–170.

HAGIWARA, S. (1983). *Membrane Potential Dependent Ion Channels in Cell Membrane. Phylogenetic and Developmental Approaches.* Raven Press, New York.

HAGIWARA, S., MIYAZAKI, S. & ROSENTHAL, N. P. (1976). Potassium current and the effect of cesium on this current during anomalous rectification of egg cell membrane of a starfish. *Journal of General Physiology* **67**, 621–638.

HAGIWARA, S. & TAKAHASHI, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *Journal of Membrane Biology* **18**, 61–80.

HAGIWARA, S. & YOSHII, M. (1979). Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *Journal of Physiology* **292**, 251–265.

HALLIWELL, J. V. & ADAMS, P. R. (1982). Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. *Brain Research* **250**, 71–92.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HILLE, B. & SCHWARZ, W. (1978). Potassium channels as multi-ion single-file pores. *Journal of General Physiology* **72**, 409–442.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium chloride ions on the membrane potential of single fibres. *Journal of Physiology* **148**, 127–160.

INOUE, M., NAKAJIMA, S. & NAKAJIMA, Y. (1988). Somatostatin induces an inward rectification in rat locus coeruleus neurones through a pertussis toxin-sensitive mechanism. *Journal of Physiology* **407**, 177–198.

KAMEYAMA, M., KIYOSUE, T. & SOEJIMA, M. (1983). Single channel analysis of the inward rectifier K current in the rabbit ventricular cells. *Japanese Journal of Physiology* **33**, 1039–1056.

KATZ, B. (1949). Les constantes électriques de la membrane du muscle. *Archives des sciences physiologique* **3**, 285–299.

KONISHI, S. & OKAMOTO, T. (1985). The relationship between substance P-mediated synaptic response and intracellular cyclic nucleotide formation in the guinea-pig inferior mesenteric ganglion. *Neuroscience Research* **1**, S102.

LEECH, C. A. & STANFIELD, P. R. (1981). Inward rectification in frog skeletal muscle fibres and its dependence on membrane potential and external potassium. *Journal of Physiology* **319**, 295–309.

LEIFER, D., LIPTON, S. A., BARNSTABLE, C. J. & MASLAND, R. H. (1984). Monoclonal antibody to Thy-1 enhances regeneration of processes by rat retinal ganglion cells in culture. *Science* **224**, 303–306.

MASUKO, S., NAKAJIMA, Y., NAKAJIMA, S. & YAMAGUCHI, K. (1986). Noradrenergic neurons from the locus coeruleus in dissociated cell culture: culture methods, morphology and electrophysiology. *Journal of Neuroscience* **6**, 3229–3241.

MIHARA, S., NORTH, R. A. & SURPRENANT, A. (1987). Somatostatin increases an inwardly rectifying potassium conductance in guinea-pig submucous plexus neurones. *Journal of Physiology* **390**, 335–355.

NAKAJIMA, Y., NAKAJIMA, S. & INOUE, M. (1988). Pertussis toxin-insensitive G protein mediates substance P-induced inhibition of potassium channels in brain neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 3643–3647.

NAKAJIMA, Y., NAKAJIMA, S., LEONARD, R. J. & YAMAGUCHI, K. (1986). Acetylcholine raises excitability by inhibiting the fast transient potassium current in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences of the USA* **83**, 3022–3026.

NAKAJIMA, Y., NAKAJIMA, S., OBATA, K., CARLSON, C. G. & YAMAGUCHI, K. (1985). Dissociated cell culture of cholinergic neurons from nucleus basalis of Meynert and other basal forebrain nuclei. *Proceedings of the National Acadamy of Sciences of the USA* **82**, 6325–6329.

NICOLL, R. A., SCHENKER, C. & LEEMAN, S. E. (1980). Substance P as a transmitter candidate. *Annual Review of Neuroscience* **3**, 227–268.

NILSSON, G., HÖKFELT, T. & PERNOW, B. (1974). Distribution of substance P-like immunoreactivity in the rat central nervous system as revealed by immunohistochemistry. *Medical Biology* **52**, 425–427.

OHMORI, H. (1978). Inactivation kinetics and steady-state current noise in the anomalous rectifier of tunicate egg cell membranes. *Journal of Physiology* **281**, 77–99.

O'LAGUE, P. H., POTTER, D. D. & FURSHPAN, E. J. (1978). Studies on rat sympathetic neurons developing in cell culture. I. Growth characteristics and electrophysiological properties. *Developmental Biology* **67**, 384–403.

OTSUKA, M., KONISHI, S., YANAGISAWA, M., TSUNOO, A. & AKAGI, H. (1982). Role of substance P as a sensory transmitter in spinal cord and sympathetic ganglia. In *Substance P in the Nervous System. Ciba Foundation Symposium*, vol. 91, pp. 13–34. Pitman, London.

SAKMANN, B., NOMA, A. & TRAUTWEIN, W. (1983). Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature* **303**, 250–253.

SAKMANN, B. & TRUBE, G. (1984). Voltage-dependent inactivation of inward-rectifying single-channel currents in the guinea-pig heart cell membrane. *Journal of Physiology* **347**, 659–683.

SEGAL, M. & BARKER, J. L. (1984). Rat hippocampal neurons in culture: Potassium conductances. *Journal of Neurophysiology* **51**, 1409–1433.

STANDEN, N. B. & STANFIELD, P. R. (1978). A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fibres by barium and strontium ions. *Journal of Physiology* **280**, 169–191.

STANDEN, N. B. & STANFIELD, P. R. (1979). Potassium depletion and sodium block of potassium currents under hyperpolarization in frog sartorius muscle. *Journal of Physiology* **294**, 497–520.

STANFIELD, P. R. (1970). The differential effects of tetraethylammonium and zinc ions on the resting conductance of frog skeletal muscle. *Journal of Physiology* **209**, 231–256.

STANFIELD, P. R., NAKAJIMA, Y. & YAMAGUCHI, K. (1985). Substance P raises neuronal membrane excitability of reducing inward rectification. *Nature* **315**, 498–501.

STANFIELD, P. R., STANDEN, N. B., LEECH, C. A. & ASHCROFT, F. M. (1981). Inward rectification in skeletal muscle fibres. In *Advance in Physiological Sciences*, vol. 5, *Molecular and Cellular Aspects of Muscle Function*, ed. VARGER, E., KÖVER, A., KOVÁCS, T. & KOVÁCS, L., pp. 247–262. Pergamon Press, New York.

YANAGIHARA, K. & IRISAWA, H. (1980). Inward current activated during hyperpolarization in the rabbit sinoatrial node cell. *Pflügers Archiv* **385**, 11–19.